The Induction of Cardiac Hypertrophy in Mice and the Subsequent Protein Analyses of the Heart Tissue

Senior Honors Thesis (HONRS 499)

by

Kristin L. Abels

Thesis Advisor
Susan A. McDowell

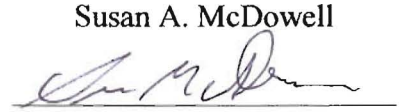

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TABLE OF CONTENTS

Abstract

Heart disease is the number one killer in the United States. Because this disease is such a prevalent killer, it is necessary to develop a better understanding of the protein pathways involved in the development of pathologic hypertrophy of the heart, which can lead to heart disease. Pathologic hypertrophy is an unhealthy type of enlargement of the tissue and arises from a pressure overload in the heart. Physiologic hypertrophy is a healthy type of hypertrophy and arises from a volume overload in the heart. The difference in the protein pathways involved in these two types of hypertrophy remains largely unknown. Perhaps there is a way to manipulate the proteins in a diseased heart to imitate physiologic hypertrophy in order to block the progression of heart disease. In this research project, a swim-training model was developed to induce cardiac hypertrophy in mice. Heart weight to body weight ratios (HW:BW) were collected for all of the hearts. Homogenates of these hypertrophied hearts as well as those of a control group were used for protein analysis. The difference in the phosphorylation state of Akt (a protein kinase involved in cell survival and cell growth) was determined for the control vs. swim-trained mice. The difference in the phosphorlyation state of p70S6K (a protein that regulates cell growth and plays a role in the pathway to determination of cell size) also was determined for the control vs. swim-trained mice.

Introduction

Cardiovascular disease (CVD) claims more lives each year than the next four leading causes of death combined *(21)*. Many people associate this disease with men, but it is also the number one killer of women, claiming nearly 500,000 lives of women a year *(8)*. Cardiovascular diseases are predicted to be the most common causes of death in the world by 2020 *(2)*. Because it is such a prevalent killer with direct treatment costs estimated at ~$21.4 billion a year *(5)*, it is of clinical importance to understand what may cause this disease. Certain protein families are known to play a role in hypertrophy (enlarged growth) of the heart, seen in patients with heart disease. One of these families is phosphoinositide 3-kinase (PI3K). PI3K is a family of related lipid kinases that phosphorylate the 3' position hydroxyl (-OH) group of the inositol (cyclic polyalcohol) ring of either phosphatidylinositol 4-monophosphate or phosphatidylinositol 4,5-bisphosphate to create phosphatidylinositol 3,4-bisphosphate (PIP_2) and phosphatidylinositol 3,4,5-triphosphate (PIP_3), respectively *(5, 6, 12)*. PI3K has an effect on many cellular responses such as proliferation, adhesion, cell size, and apoptosis *(2)*.

There are three classes that make up the PI3K family, with class I being the most widely understood *(6)*. Class I PI3K's are heterodimers made up of a regulatory subunit (p85) and a catalytic subunit (p110) consisting of four isoforms (α, β, γ, δ). The p110 α, β, and δ isoforms are activated by receptor tyrosine kinase pathways, whereas the γ and β forms are activated by the βγ subunit of G-proteins and act downstream of G-coupled protein receptors (GPCRs) *(2, 27)*. Little information is known about the role of class II and III PI3Ks in cardiac tissue *(6)*.

The p110α isoform of PI3K has been found to play a significant role in the induction of physiologic (healthy) hypertrophy *(3)*, while the p110γ isoform can cause pathologic (unhealthy) hypertrophy *(30)*. Physiologic hypertrophy is characterized by normal cardiac structure,

preserved or improved heart function, and little change in the gene expression pattern, whereas pathologic hypertrophy leads to tissue damage, and deterioration in heart function, which may ultimately result in heart failure *(5)*. When the heart experiences an increased wall stress, it compensates with this pathologic growth *(2)*.

When PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate to create PIP_3, it activates several downstream effectors. Three of these effectors are Akt, mammalian target of rapamycin (mTOR), and p70 ribosomal S6 kinase (p70S6K). Akt activates mTOR, and mTOR activates p70S6K. Akt is a protein kinase that regulates metabolism, protein translation, gene transcription, and cell survival *(13)*. mTOR is a protein that plays a role in the pathway to determination of cell, organ, and body size *(10)*. p70S6K is a kinase that phosphorylates the 40S ribosomal S6 protein.

Understanding the Pathway

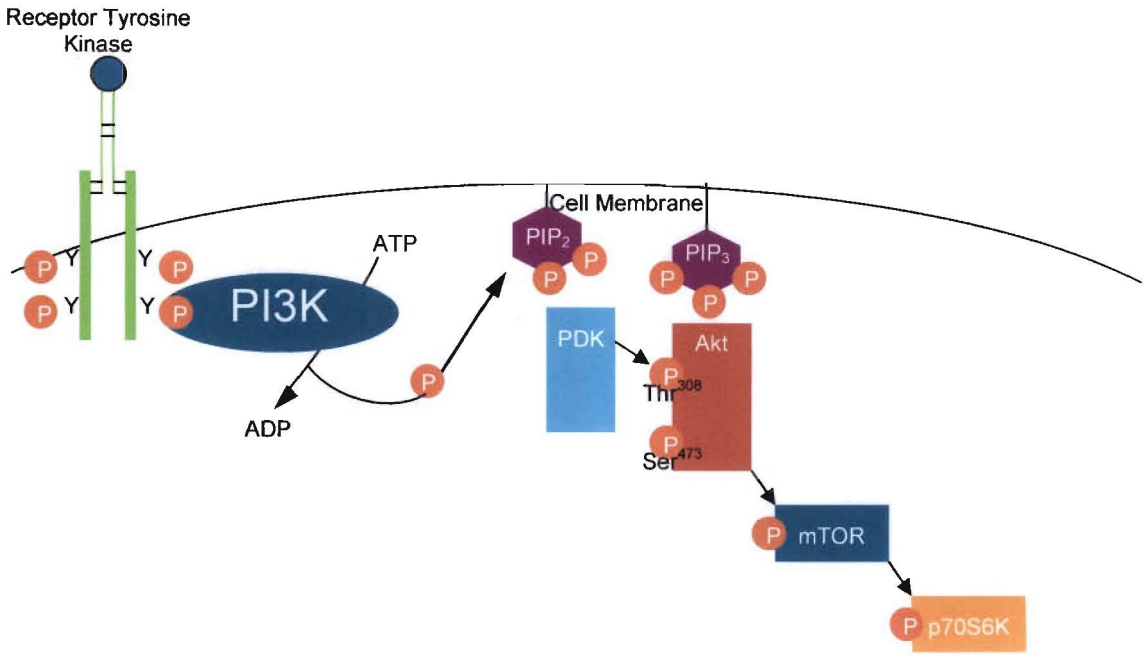

Figure 1. Diagrammatic depiction of one of the phosphoinositide 3-kinase (PI3K) pathways. Once a ligand binds to a receptor tyrosine kinase, specific tyrosine residues within the receptor are auto-phosphorylated. PI3K recognizes this auto-phosphorylation and hydrolyzes ATP. PI3K then goes on to phosphorylate the 3' –OH group of phosphatidylinositol 4,5-bisphosphate to create phosphatidylinositol 3,4,5-triphosphate (PIP_3). Phosphoinositide-dependent kinase (PDK) and protein kinase B (also called Akt) are two other proteins that recognize the activated PIP_3 molecules. Because of the close proximity of PDK and Akt, PDK is able to phosphorylate Akt. Once Akt is activated, it is released from the PIP_3 molecule. Akt then phosphorylates mTOR, which in turn phosphorylates a 70 kDa protein kinase, p70S6K.

In this study, two downstream effectors of PI3K were studied: Akt and p70S6K. Protein kinase B (PKB or Akt) is a serine/threonine kinase that mediates several cellular responses to external stimuli *(11)*. Akt substrates are found everywhere in the cell. However, Akt normally accumulates in the nucleus *(13)*.

Akt binds to the products of PI3K, PIP_2 and PIP_3, with its pleckstrin homology domain (a protein region of approximately 120 amino acids that can bind phosphatidylinositol lipids in biological membranes) *(12)*. 3-phosphoinositide-dependent kinases, PDK1 and PDK2, phosphorylate Akt at the threonine 308 residue and the serine 473 residue, which activates this kinase.

Akt promotes protein synthesis in many ways. For example, it phosphorylates glycogen synthase kinase 3β (GSK-3β), which leads to the inhibition of this synthase and the upregulation of protein synthesis *(14)*. Akt also indirectly can activate the kinase, p70S6K, through phosphorylation of mTOR. mTOR then phosphorylates p70S6K, which activates p70S6K and allows it to promote protein synthetic pathways.

The second protein studied in this experiment was the 70-kDa ribosomal protein S6 kinase (p70S6K). p70S6K is a serine/threonine kinase also involved in the regulation of protein synthesis *(15)*. This kinase is a dual pathway kinase *(16)*. p70S6K signals cell survival through a pro-apoptotic protein substrate known as mitochondrial BAD. p70S6K also signals cell growth through a different substrate, the ribosomal subunit S6.

p70S6K is able to control cell size and has been found to contribute significantly to tumor development *(17)*. p70S6K is active in cell cycle control, neuronal cell differentiation, regulation of cell motility, the immune response, and tissue repair. This kinase is involved in metabolic diseases and disorders such as obesity, diabetes, insulin resistance, and hyperglycemia

(18).

The PI3K pathway affects cardiac hypertrophy. Therefore, finding drug treatments to negate the effects of these proteins might be able to decrease the number of people that are harmfully affected by heart disease. Crackower and Penninger have patented a pharmaceutical composition that is an inhibitor of PI3Kγ, and aids in treating heart disease *(2)*. This pharmaceutical compound is selected from the group consisting of PI3K inhibitors wortmannin, LY294002, and quercetin and their derivatives. The pharmaceutical composition affects the PTEN/ PI3Kγ pathway. PTEN is a lipid phosphatase that dephosphorylates the 3' –OH position of PIP_3, and thus has been identified as the master regulator for heart size and heart function. Mice that were genetically altered to be dominant negative for PTEN developed hypertrophy of the heart. They found that inhibition of PI3Kγ and/or activation of PTEN has an opposite effect in that it blocks cardiac hypertrophy. These findings imply future directions for further study since Crackower and Penninger have found a novel method of treating heart disease.

Oudit also studied the effects of PI3Kγ seen in heart disease *(7)*. The results from this study provide the first genetic evidence for a potential therapeutic role, the inhibition of PI3Kγ, in treating heart failure. The treatment would include controlling the level of PI3Kγ signaling that occurs in the cells. Pointing to future directions of study, Oudit indicates that the role of PI3Kγ inhibition in other genetic and acquired models of heart failure and the reduction in interstitial fibrosis (scarring that occurs in the interstitium) need to be evaluated further.

Connolly has patented certain chemical compounds, known as cycloalkyl and heterocycloalkyl substituted benzothiophenes, to help treat PI3K-mediated disorders. PI3K-mediated disorders are characterized by the participation of one or more of the PI3K isoforms or a PIP_3 phosphatase (e.g., PTEN) in the progression of a disorder or condition such as

cardiovascular diseases, atherosclerosis, hypertension, rheumatoid arthritis, cancer, or type II diabetes *(1)*. This treatment method is in the form of a syrup, an elixir, a suspension, a powder, a tablet, a capsule, an ointment, or a lotion. The compounds of the present invention could be used to treat PI3K-mediated disorders preventatively, acutely, or chronically. This patented compound can be co-administered with other compounds that are useful for treating heart disease, such as aspirin.

Robertson also has patented several compounds that inhibit PI3K and provide anti-thrombotic activity amongst many other pharmaceutical properties. The morpholino-substituted pyridopyrimidine, quinolone, and benzopyranone derivatives have been useful in treating PI3K-dependent conditions including cardiovascular diseases such as stroke, coronary artery occlusion, acute myocardial infarction, and many others *(9)*.

McMullen has found yet another potential treatment for heart failure, the insulin-like growth factor 1(IGF1). Aortic banded hearts of transgenic mice that displayed an over-expression of the IGF1 receptor had significantly less interstitial fibrosis than aortic banded non-transgenic mice, which suggests that this receptor might play a protective role in heart disease. The results from this study indicate that the IGF1-PI3K(p110α) pathway plays an important role for promoting physiologic hypertrophy and improving contractile function *(4)*.

In addition to the PI3K treatments, there have been a few drug treatments developed that inhibit the actions of p70S6K as well. Rapamycin (an immunosuppressant macrolide) is a drug that inhibits mTOR, and, in turn, p70S6K *(10)*. These results suggest that a useful therapeutic agent, rapamycin, may suppress cardiac hypertrophy in patients. Another drug, patented by Davies, uses pyrazole-containing aryl- and heteroaryl-alkylamine compounds in the treatment or prevention of a disease in which the modulation (inhibition) of p70S6K is indicated *(18)*. This

drug treatment can be used to inhibit tumor metastasis, bladder cancer, ovarian cancer, congestive heart failure, hypertension, renal dysfunction, atherosclerosis, and cardiac hypertrophy.

Several treatments also have been found that deal with cardiac hypertrophy in general. Modulation (upregulation) of the integrin-linked kinase (ILK) signaling pathway has been found to enhance the remodeling process relevant to a large number of cardiac diseases. Overexpression of ILK aids in the process for mediating a broadly adaptive form of human cardiac hypertrophy and a protective process for post myocardial infarction *(19)*. Isorhapontigenin (ISO), a new resveratrol analog, lessens cardiac hypertrophy by blocking oxidative stress and oxidative stress-mediated pathways *(20)*. ISO acts by blocking the PI3K-Akt-GSK3β/p70S6K pathway, preventing cardiac hypertrophy through an antioxidant mechanism involving inhibition of different intracellular signaling transduction pathways.

Treatment options, however, are far from being exhausted. As the number one killer in the United States, one in three Americans has some form of CVD *(21)*. This statistic in itself indicates that research in this field will be an on-going process. Perhaps, in the future, scientists will be able to synthesize other drug treatments such as a very efficient inhibitor of PI3Kγ or activator of PI3Kα. PI3Kα plays a critical role in inducing physiological hypertrophy *(5)*. In transgenic mice expressing a constitutively active PI3Kα mutant, PI3K activity was elevated 6.5-fold, heart size increased, cardiac function was normal, and there was no evidence of fibrosis. Therefore, an inhibitor of PI3Kγ and/or an activator of PI3Kα may help to decrease the number of citizens suffering from heart disease.

Because this disease is so prevalent, we thought it would be beneficial to take on a research project of our own. This research process began by meeting with Dr. McDowell during

my freshman year with the sole purpose of reading specific journal articles written on topics such as PI3K, Akt, p70S6K, IGF1, Rapamycin, and their roles in cardiac hypertrophy.

After gaining a better understanding of the pathways involved in the hypertrophy process, we developed a swim-training model for mice during my sophomore year, modifying the method found in *(22)*. For model number one, one group of female BALB/c mice were swum 10 minutes twice a day with an intent to increase up to 90 minutes twice a day, while a second group remained sedentary. The mice were swum in water that was only room temperature, causing many of them to die from hypothermia and thus the experiment was terminated. For model number two, one group of female C57BL/6 mice were swum twice daily for a month, starting at five minute intervals and working up to 90 minutes per swim, while a second group remained sedentary. A heated water bath was used for the swim trainings. The water temperature was recorded daily with an average of 30°C. Heart weight to body weight ratio (HW:BW) was determined for both groups of mice because this ratio is a common way of measuring hypertrophy of the heart. There was no significant difference in HW:BW between the two groups of mice, so swim-model number three was developed. In this final model, one group of male C57BL/6 mice were swum according to model number two, while a second group remained sedentary. A significant increase was observed in HW:BW for the swim-trained mice. Therefore this model was used for subsequent protein analyses.

To investigate further the effects of swim-training on a cellular level, we decided to study in detail two specific proteins within the PI3K pathway, Akt and p70S6K. The heart tissue was homogenized in order to determine the protein concentrations in each heart and to assess by western blot analysis. The samples were boiled and placed in a loading buffer containing a reducing agent, dithiothreitol (DTT), and sodium dodecyl sulfate (SDS) in order to denature the

polypeptide chains. DTT reduces disulfide bonds to make the proteins linear. SDS coats the proteins with a negative charge. The proteins were then separated based on size with a technique known as polyacrylamide-gel electrophoresis (PAGE) *(23)*. This process utilizes gels containing SDS to coat the proteins with a negative charge. The proteins are then attracted to the positively charged electrode in the electrophoresis device, allowing the proteins of lower molecular weight to travel the furthest through the gel. As the percentage of acrylamide gel increases, the proteins experience a greater resistance to travel through the gel. Therefore higher percentage gels are used to separate out smaller proteins. Proteins of known molecular weight also are included in one lane of the gel in order to compare the distance traveled by the proteins of interest (Akt and p70S6K) to the distance traveled by the proteins of known molecular weight. When graphing the log of the molecular weight of the marker proteins vs. the distance migrated through the gel, a linear function is produced.

In our research project, a NuPAGE 4-12% Bis-Tris Gel was used with MES [2-(*N*-morpholino)ethanesulfonic acid] buffer. NuPAGE gels provide the best separation and resolution of small- to medium-sized proteins by using a neutral pH environment *(28)*. This type of environment minimizes protein modifications such as deamination and alkylation. The original Laemmli system cast gels at a pH of 8.4, causing the gel to have a shelf-life of one-two months. Because the NuPAGE Bis-Tris Gels are cast at a pH of 6.4, they have a much longer shelf life of 12 months. Compared to the Laemmli system, the NuPAGE system provides better protein stability during the run of the gel. The NuPAGE system also allows for higher protein capacity with excellent band resolution, most efficient transfers, and fastest run times. The optimized buffers of the NuPAGE system provide complete reduction of disulfide bonds under mild conditions and avoid protein cleavage during sample preparation. NuPAGE MES and NuPAGE

MOPS [3-(N-morpholino)propanesulfonic acid] Running Buffers are used with NuPAGE Bis-Tris Gels (Bis-Tris 10%, 12%, and 4-12%). Each buffer creates a different migration pattern when used with the same gel. Therefore, six different types of migration patterns can be formed when combining the three types of gels with the two buffers. The NuPAGE MES buffer is recommended for resolving small proteins, while NuPAGE MOPS buffer is recommended for resolving medium to large size proteins.

After separating the proteins based on molecular weight, the next step is to transfer these proteins from the polyacrylamide gel to a membrane *(24)* through a process known as western blotting. Polyvinylidene fluoride (PVDF) and nitrocellulose are the two most common types of membranes used in this type of procedure *(25)*, with PVDF being used in this research project. The PVDF membrane is sandwiched with the gel in between layers of filter paper soaked in transfer buffer, which allows the transfer of proteins from the gel to the membrane. The assembly is then placed into an electrophoretic chamber with the membrane facing the negative electrode *(24)*. A voltage is applied to the chamber for 30 minutes in order to transfer the proteins from the gel to the membrane.

The membrane is then probed with antibodies in order to visualize the proteins of interest. The primary antibody is used to recognize and bind to the protein of interest. A secondary antibody is also used, which is raised against the species of the primary antibody. This secondary antibody recognizes and binds to the primary antibody, and is conjugated with a flourophore molecule. To create the antibodies, the basal protein of interest (e.g. Akt or p70S6K) is injected into a mouse. The mouse then makes antibodies for this protein, which are also called immunoglobulins (IgG). The mouse IgG (primary antibody) is then injected into a goat. The goat forms goat anti-mouse IgG (secondary antibody), which is then conjugated with a fluorophore

molecule. The phosphorylated protein of interest (e.g. phospho-Akt or phospho-p70S6K) is injected into a rabbit. The rabbit makes antibodies for the phosphorylated protein. The rabbit IgG is then injected into a goat. The goat forms goat anti-rabbit IgG, which is also conjugated with a fluorophore molecule to allow the protein to fluoresce at a particular wavelength when using the LI-COR Odyssey Infrared Imager laser scanner *(26)*. Primary antibodies were raised in two different species in order to differentiate between the phosphorylated protein and the basal protein. The basal protein fluoresces green when a wavelength of 800 nm is used. The phosphorylated protein fluoresces red when a wavelength of 680 nm light is used.

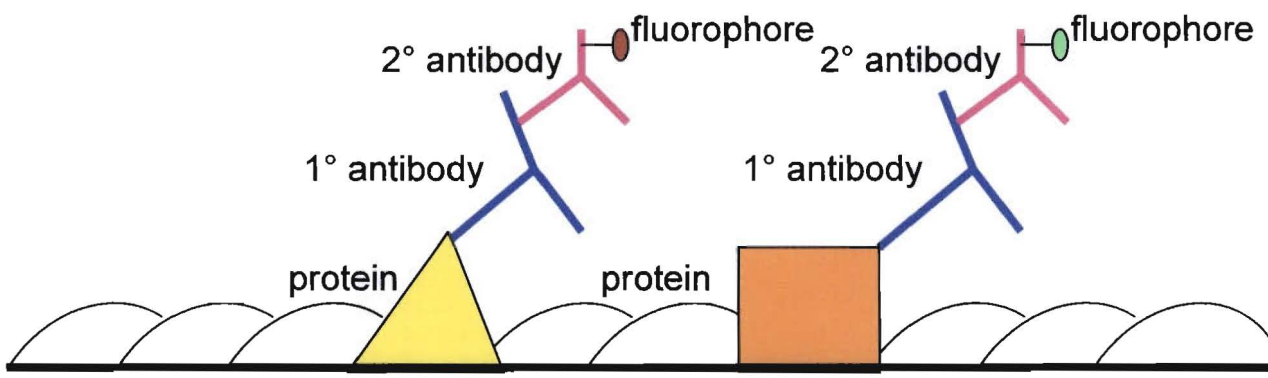

Figure 2. Antibody probing. The solid black, horizontal line represents the PVDF membrane. The primary antibodies recognize and bind to a specific epitope of the protein. The secondary antibodies are raised against the primary antibodies and are able to recognize and bind the primary antibodies. The secondary antibodies are conjugated with a fluorophore which fluoresces when scanned with a laser. Basal protein fluoresces as a green band and phosphorylated protein fluoresces as a red band.

The objective of the current study was to determine whether or not the phosphorylated form of Akt and p70S6k were expressed at a higher level compared to their basal forms. A higher level of phosphorylation of Akt and/or p70S6K for the swim-trained mice may indicate that the hypertrophied heart tissue was modified in some way. These protein modifications seen in physiologic hypertrophy in the swim-trained mice could be related to the process seen in pathologic hypertrophy, potentially providing opportunities for discovering new drug treatments for treating heart disease.

Materials and Methods

Swim-training model #1. Eight female BALB/c mice were swum in a tank of room-temperature water for 10 minutes, twice daily with an intent to increase up to 90 minutes a day. A second group of eight female BALB/c mice remained sedentary. Many of the mice died due to hypothermia and the experiment was terminated.

Swim-training model #2. Nine female C57BL/6 mice were swum in 30-31°C tank for five minutes, twice daily for one week. The mice were swum for 10 minutes, twice daily for two days, 20 minutes, twice daily for two days, increasing in 10 minute increments until 90 minutes was achieved and sustained for three-four weeks (See Table 1). One group of seven female C57BL/6 mice remained sedentary. Four swim-trained mice died throughout the experiment. The remaining mice were put to death via CO_2 gas, and body weight was measured. The mice were exsanguinated. Hearts were extracted, rinsed in phosphate buffer solution (PBS), and weighed. No statistical significance was observed in HW: BW in swim vs. control mice.

Day	1	2	3	4	5	6	7	8	9	10	11
AM swim (min)	5	5	5	5	5	10	10	20	20	30	30
PM swim (min)	5	5	5	5	5	10	10	20	20	30	30
Day	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**
AM swim (min)	40	40	50	50	60	60	70	70	80	80	90
PM swim (min)	40	40	50	50	60	60	70	70	80	80	90
Day	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**
AM swim (min)	90	90	90	90	90	90	90	90	90	90	90
PM swim (min)	90	90	90	90	90	90	90	90	90	90	90
Day	**34**	**35**	**36**	**37**	**38**	**39**	**40**	**41**	**42**	**43**	**44**
AM swim (min)	90	90	90	90	90	90	90	90	90	90	90
PM swim (min)	90	90	90	90	90	90	90	90	90	90	90

Table 1. Swim-training model for C57BL/6 mice. Mice began swimming with five minute intervals twice a day for five days. The mice were swum for 10 minutes twice daily for two days, increasing in 10 minute increments until 90 minutes was achieved and sustained for three-four weeks.

Swim-training model #3. Fifteen male C57BL/6 mice were swum according to model #2. One group of six male C57BL/6 mice remained sedentary. Nine swim-trained mice died throughout the experiment. HW: BW was determined for the remaining six swim-trained mice and six sedentary mice. This model was used for subsequent protein analyses because a statistical significance in HW: BW was observed.

Tissue Homogenization. 250ml of Lysis Buffer was prepared for homogenization (20mM Trizma, Cat# T-1378; 137mM NaCl, Cat# S7653; 1mM $MgCl_2$-$6H_2O$, Cat# 5980; 1mM $CaCl_2$, Cat# 3000; 10% glycerol; 1% NP40). HCl was added to the solution to bring down the pH to 7.5. The solution was filtered to remove any bacteria. One Complete Mini-tab (Roche 1836153) and 40µl microcystin at 0.5mg/ml were added to 10ml of Lysis Buffer. The Complete Mini-tab is a protease inhibitor, while microcystin is a phosphatase inhibitor. 1.5ml of Lysis Buffer was added to a 4ml tube which contained the mouse heart. A tissue mizer (blender) was used to blend each heart for 45 seconds. The hearts were homogenized in a cold room (4°C) to slow down the process of proteases, which break down proteins.

Protein concentration determination. After homogenization, the samples were immediately placed on ice. The heart homogenates were rocked at 4°C for 20 minutes. Two, 1ml portions from each of the 12 samples were aliquoted into 1.5ml microcentrifuge tubes. The samples were spun in a centrifuge at 4°C at 14,000 rpm for 20 minutes. The supernatant was transferred to cold microcentrifuge tubes, which were well labeled. Three dilutions of 1:10 were made for each sample. A 96-well plate was loaded with 8µl of each sample (see Table 2).

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank											
B	13c	13c	13c	14c	14c	14c	15c	15c	15c	16c	16c	16c
C	17c	17c	17c	18c	18c	18c	26s	26s	26s	27s	27s	27s
D	28s	28s	28s	29s	29s	29s	30s	30s	30s	31s	31s	31s
R												
F												
G	.0625	.0625	.0625	.125	.125	.125	.25	.25	.25	.50	.50	.50
H	1.0	1.0	1.0									

Table 2. Samples in the 96-well plate. All samples are 1:10 dilutions. "13c" – "18c" are the six control mice samples. "26s" –"31s" are the six swim-trained mice samples. Rows G and H are the standard bovine serum albumin (BSA) concentrations in mg/ml.

A repeat pipettor was used to place 200µl of Coomassie stain into each well. Coomassie stains the proteins. A 2ml pipet was used to blow 95% ethanol vapor over the wells to break any bubbles that were present. The BioRad Model 680 Microplate Reader was used to read the 96-well plate. This device is a spectrophotometer that makes a standard curve from the known bovine serum albumin (BSA) protein concentrations and then reads the absorbance from the heart homogenates. A standard curve was made using BSA concentrations, and protein concentrations were determined for each heart homogenate.

Polyacrylamide gel electrophoresis. 1X running buffer was made from NUPAGE MES 20X Running Buffer (cat# NP0002). To 90µl of 4X NP LDS Sample Buffer (cat # NP0007), 10µl of NP Sample Reducing Agent (cat# NP0009) was added to make 4X Loading Buffer.

80µg of each sample was added to the lysis buffer to make a final volume of 24µl. To this mixture, 8µl of 4X Loading Buffer was added. The samples were then heated for five minutes at 90°C and placed in the VWR centrifuge for a quick spin (14,000 rpm for 10 seconds). 32µl of each sample was loaded into the gel. 20µl of See Blue Plus2 pre-stained standard ladder (cat# LC5925) was loaded into lane one of the gel as a standard to which the samples were compared. Running Buffer was poured into the middle chamber over the top of the well in order for the current to be able to run through the entire gel. Running Buffer was also poured into the outer chamber as a cooling chamber. Running Buffer acts like an electrolyte, creating the electric current. An Invitrogen NuPAGE 4-12% Bis-Tris Gel (cat# NP0321 Box) was used for this process. The electrophoresis apparatus was connected to a BioRad Power supply, which was set at 200V for 35 minutes.

Western Blot. Transfer Buffer was prepared by mixing 50ml NuPAGE Transfer Buffer (20X) with 850ml milli-Q water and 100ml methanol. Once the gel was finished running, the gel was placed in transfer buffer while sitting on a rocker. Two blotting pads were saturated with transfer buffer and placed in the box portion of the Western blot apparatus. One layer of the PVDF membrane filter paper (cat# LC2002) was placed on top of the blotting pads after soaking it in transfer buffer. The gel was picked up (using gloves) and was placed on top of the filter paper. The transfer membrane was saturated with methanol and immediately placed on top of the gel. A glass test tube was rolled over the membrane to push out any bubbles. The outer layer of the membrane filter paper was soaked in transfer buffer and placed on top of the membrane. Several more blotting pads were soaked in transfer buffer and placed on top of the filter paper until the box was completely full. The lid was placed on the box and the apparatus was placed in the chamber. The chambers were filled with transfer buffer, which allows for the proteins to transfer

from the gel to the membrane. The Western was run for 30 minutes at 60V.

Blocking. The PVDF membrane was re-wetted in 100% methanol, rinsed twice in milli-Q water, and once in 1X PBS (cat# 20012-043) to rinse off any extra methanol. The membrane was then placed directly into 50ml of Odyssey Blocking Buffer (cat# 927-40,000) for one hour with shaking. This buffer blocks the nonspecific proteins so that the antibody can find the antigen efficiently.

Probing. The primary antibodies used were: Akt1 (cat# 2976), phospho-Akt (cat# 9271), p70S6K (cat# S87620), and phospho-p70S6K (cat# 9205S). The primary antibodies were diluted (1:1000). First, 0.1% Tween-20 was added to 40ml of Odyssey Blocking Buffer. The desired dilution of the antibody was then added to the mixture of Tween-20 and Odyssey Blocking Buffer. The diluted antibody solution was then added to the membrane and placed in the 4°C fridge to rock for the week. The antibody solution was poured off. The membrane was washed once very briefly in 1X PBS + 0.1% Tween-20 at room temperature, and washed again, twice for five minutes each. The appropriate amount of diluted secondary antibody (1:40,000) was added to 40ml Odyssey Blocking Buffer and 0.1% Tween-20. The secondary antibodies used were: Akt goat anti-mouse (cat# 610-131-121), phospho-Akt goat anti-rabbit (cat# A21076), p70S6K goat anti-mouse (cat# 610-131-121), and phospho-p70S6K goat anti-rabbit (cat#A21076). The membrane was then probed with the diluted secondary antibody solution and rocked at room temperature for one hour. The membrane was protected from light for this step and all remaining steps. The secondary antibody solution was poured off. The membrane was washed once very briefly in 1X PBS + 0.1% Tween-20 at room temperature, and then twice, five minutes each. The membrane was rinsed in PBS to remove residual Tween-20.

Scanning. The standard molecular weight markers were marked with pencil. The inverted blot was placed in the lower left-hand corner of the LI-COR Odyssey Infrared Imager screen. The rubber cover was placed on the membrane. A roller was used to remove any bubbles present. For Akt, the 700 channel (phosphorylated protein) was scanned at an intensity of 7.5. The 800 channel (basal protein) was scanned at an intensity of 5.0. For p70S6K both channels were scanned at an intensity of 7.5.

Statistical analysis. LI-COR Odyssey Infrared Imager was used to determine the average intensity of the protein bands on the membrane. Sigma Stat was used to run a student's t-test in order to determine if there was a significant difference between the phosphorylation states of the swim-trained vs. control mice. Sigma Plot was used to create histograms of 700/800 channel +/- SEM for the control vs. swim-trained mice. Data were considered statistically different at $p \leq 0.05$.

Results

Swim-Training model #2.

Group	Mouse #	Mouse weight (g)	Heart weight (g)	HW:BW	Observations
Control	1	24	0.096	0.00400	
Control	2	24	0.094	0.00392	
Control	3	24	0.113	0.00471	
Control	4	24	0.097	0.00404	
Control	5	24	0.102	0.00425	
Control	6	22	0.080	0.00364	
Control	7	25	0.093	0.00372	
				Avg: 0.00404	
Swim-trained	8	25	0.110	0.00440	bloody lungs
Swim-trained	9	24	0.099	0.00413	bloody lungs
Swim-trained	10	21	0.087	0.00414	bloody lungs
Swim-trained	11	21	0.095	0.00452	bloody lungs
Swim-trained	12	22	0.102	0.00464	bloody lungs
				Avg: 0.00437	

Table 3. Results of the second swim-training model. Seven control mice were weighed and five swim-trained mice were weighed. The heart weight to body weight ratio (HW:BW) was determined for each mouse.

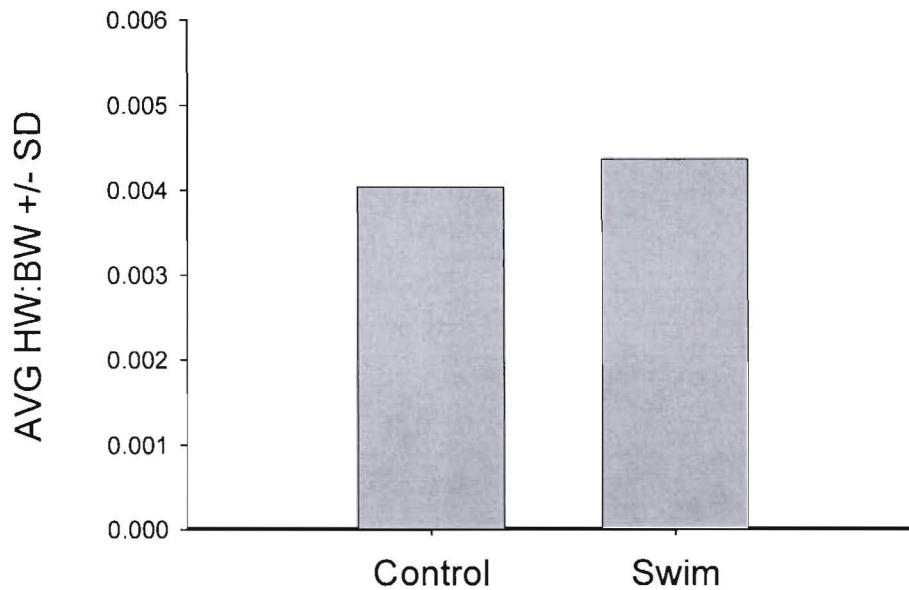

Figure 3. Histogram of the average heart weight to body weight ratio (HW:BW) of control vs. swim-trained mice for swim model number 2.

Swim-Training model #3.

Group	Mouse #	Mouse weight (g)	Heart weight (g)	HW:BW	Observations
Control	13	29	0.113	0.00390	bloody lungs
Control	14	28	0.126	0.00450	bloody lungs
Control	15	30	0.120	0.00400	bloody lungs
Control	16	29	0.117	0.00403	bloody lungs
Control	17	30	0.115	0.00383	bloody lungs
Control	18	29	0.103	0.00355	bloody lungs
				Avg: 0.00397	
Swim-Trained	26	29	0.189	0.00652	bloody lungs
Swim-Trained	27	26	0.129	0.00496	bloody lungs
Swim-Trained	28	26	0.141	0.00542	bloody lungs
Swim-Trained	29	29	0.135	0.00466	bloody lungs
Swim-Trained	30	24	0.118	0.00492	bloody lungs
Swim-Trained	31	27	0.132	0.00489	bloody lungs
				Avg: 0.00523	

Table 4. Results from swim-training model number three. Six control mice were weighed and six swim-trained mice were weighed. The heart weight to body weight ratio (HW:BW) was determined for each mouse, with a statistically significant increase in the swim-trained mice.

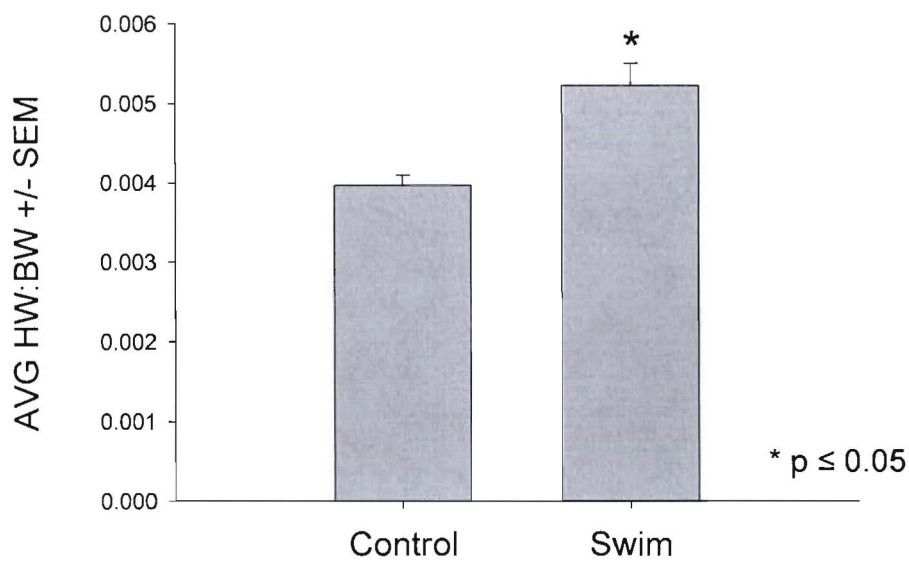

Figure 4. Histogram of the average heart weight to body weight ratio (HW:BW) of control vs. swim-trained mice for swim model number three. A statistically significant difference in the HW: BW was observed for the swim-trained mice. These hearts were used for subsequent protein analyses.

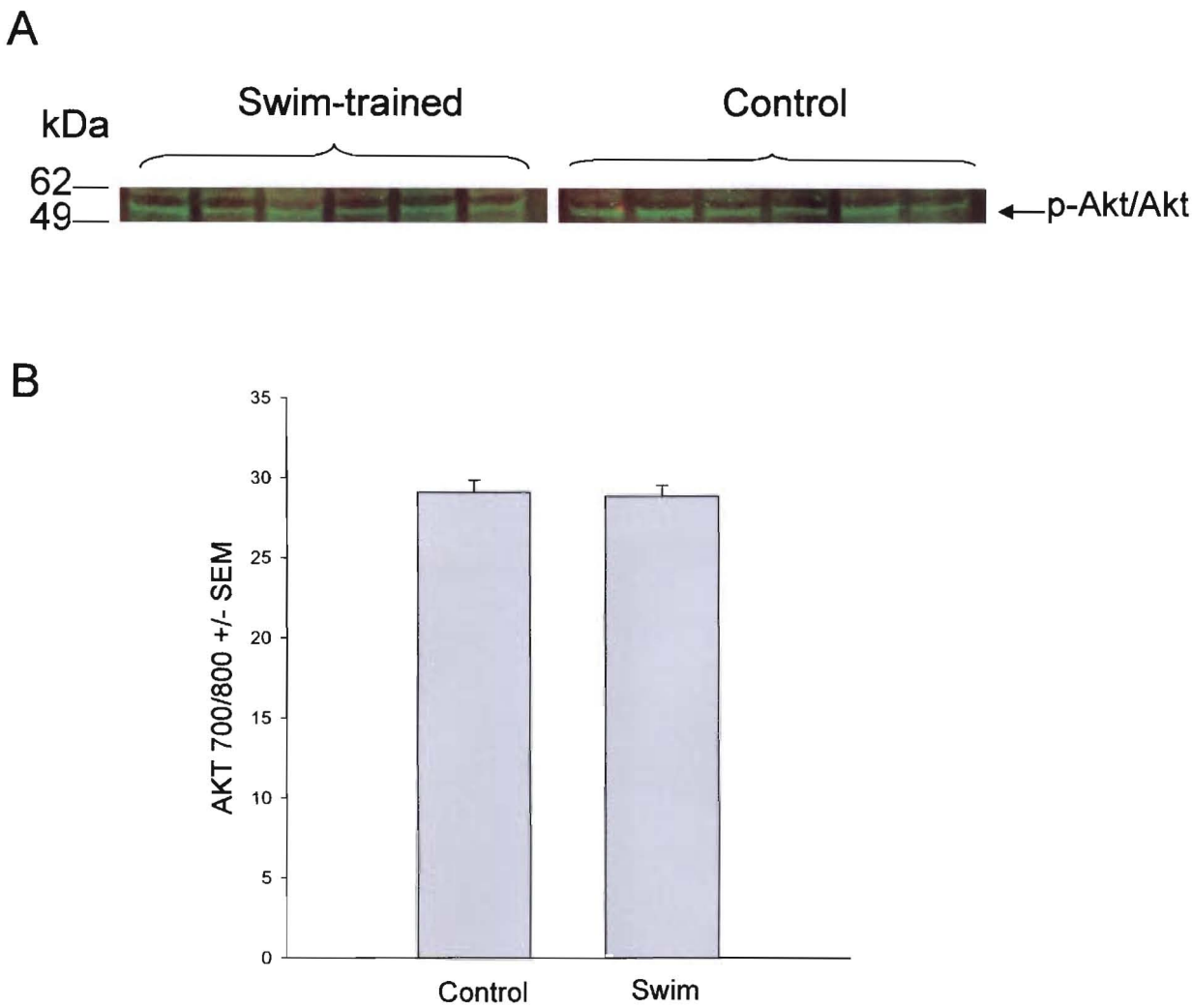

Figure 5. A)LI-COR scanning image of the PVDF membrane for Akt. Green bands represent basal protein and red bands represent phosphorylated protein. **B)** Histogram of the 700/800 channel +/- SEM for Akt in control vs. swim-trained mice. Basal Akt and phospho-Akt levels were determined for both control and swim-trained mice using average intensity data. No significant difference was observed in the phosphorylation of Akt between control and swim-trained mice.

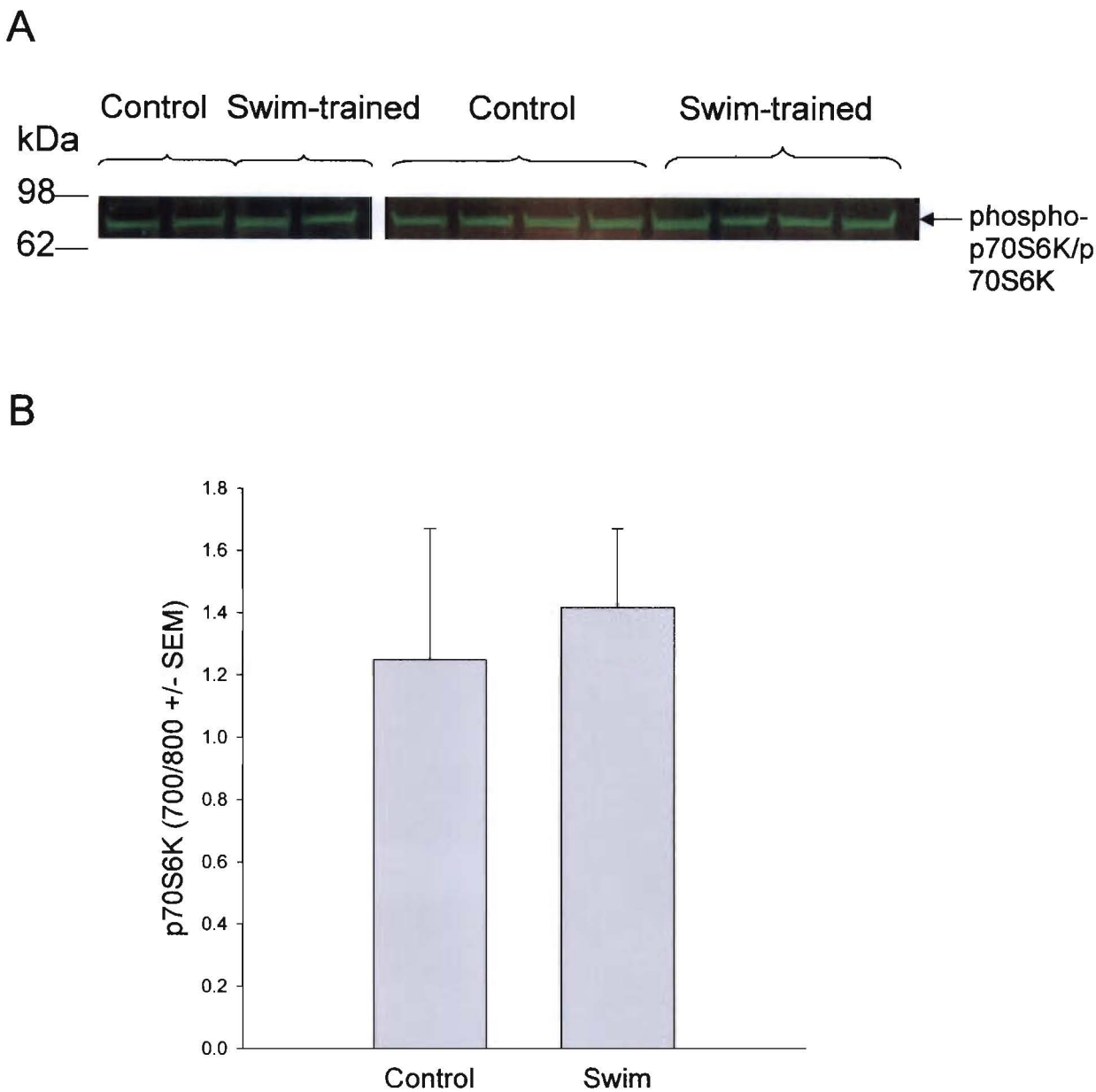

Figure 6. A) LI-COR scanning image of the PVDF membrane for p70S6K. Green bands represent basal protein and red bands represent phosphorylated protein. **B)** Histogram of the 700/800 channel +/- SEM for p70S6K in control vs. swim-trained mice. Basal p70S6K and phospho-p70S6K levels were determined for both control and swim-trained mice using average intensity data. No significant difference was observed in the phosphorylation of p70S6K between control and swim-trained mice.

The results for the second swim-training model can be found in Table 3. Five swim-trained mice survived the swim-training and were weighed. All seven control mice used in the experiment were also weighed. The entire mouse was weighed first. The heart was dissected and weighed next. Upon dissecting the heart, bloody lungs were observed in all of the swim-trained mice. HW:BW was determined for all twelve mice. The average HW:BW for the control group was 0.00404. The average HW:BW for the swim-trained group was 0.00437. The average HW:BW value for both groups was plotted on a histogram in Figure 3. There was no statistically significant difference in the HW:BW in the control vs. swim-trained groups ($p > 0.05$).

The results for the third swim-training model can be found in Table 4. Six swim-trained mice survived the swim-training model and were weighed. All six control mice used in the experiment were also weighed. The entire mouse was weighed first. The heart was weighed next. Bloody lungs were observed in all of the mice. The HW:BW was determined for both groups. The average HW:BW for the control group was 0.00397. The average HW:BW for the swim-trained group was 0.00523. The average HW:BW value for both groups was plotted on a histogram in Figure 4. There was a statistically significant increase (32%) in the HW:BW in the swim-trained group ($p \leq 0.05$). These mouse hearts were used for subsequent protein analyses.

Akt was the first protein analyzed in this study. The LI-COR Odyssey laser-scanning image for Akt can be seen in Figure 5a. Because Akt has a molecular weight of 60 kDa, it was found between the standard protein markers with molecular weights of 62 kDa (bovine serum albumin) and 49 kDa (glutamic dehydrogenase). The green band represents the basal protein. The red band represents the phosphorylated protein. Figure 5b is a histogram of the average intensities of the 700 channel divided by the average intensities of the 800 channel for the control and swim-trained mice. The 700 channel recognizes the phosphorylated protein (red bands). The

800 channel recognizes the basal protein (green bands). The higher the 700/800 ratio, the more phosphorylation was present. The 700/800 average intensity values for control mice 13, 14, 15, 16, 17, and 18 were 32, 29, 29, 29, 27, and 28, respectively. The average 700/800 value for the control group was 29. The 700/800 average intensity values for swim-trained mice 26, 27, 28, 29, 30, and 31 were 27, 30, 29, 30, 27, and 29, respectively. The average 700/800 value for the swim-trained group was 29. A student's t-test was used to determine that there was no significant difference in the phosphorylation of Akt in the control vs. swim-trained mice ($p > 0.05$).

The second protein analyzed in this study was p70S6K. The LI-COR Odyssey laser-scanning image of p70S6K can be found in Figure 6a. Because p70S6K has a molecular weight of 70 kDa, it can be found between the standard protein markers with molecular weights of 98 kDa (phosphorylase) and 62 kDa (bovine serum albumin). The green bands represent the basal protein. The red bands represent the phosphorylated protein. Figure 6b is a histogram of the average intensities of the 700 channel divided by the average intensities of the 800 channel for the control vs. swim-trained groups. The 700/800 average intensity values for control mice 13, 14, 15, 16, 17, and 18 were 0.6, 1.6, 0.5, 0.0, 2.5, and 2.3, respectively. The average intensity of the 700/800 channel for the control mice was 1.1. The 700/800 average intensity values for swim-trained mice 26, 27, 28, 29, 30, and 31 were 1.4, 0.3, 1.7, 1.4, 2.2, and 1.5, respectively. The average intensity of the 700/800 channel for the swim-trained mice was 1.4. A student's t-test was used to determine that there was no significant difference in the phosphorylation of p70S6K in the control vs. swim-trained mice ($p > 0.05$).

Discussion

Enlarged growth of the heart is a major cause of morbidity and mortality worldwide *(20)*. Pathologic (unhealthy) hypertrophy leads to heart disease, which is the number one killer in the United States *(21)*. Because heart disease is so prevalent in the USA and worldwide, the goal of this project was to develop a better understanding of the proteins involved in enlargement of the heart. Ultimately, if the results of this project were significant, a connection could be made between the pathways involved in physiologic (healthy) hypertrophy and in pathologic hypertrophy seen in heart disease.

Swim-training models were modified from *(5, 22)* in order to study the proteins involved in physiologic hypertrophy. In Kaplan's study (1994), groups of 12 eight-week-old female C57/BL6 mice were made to swim in tanks with surface area of 225 cm^2 and a depth of 15 cm with a water temperature of 30-32 °C *(22)*. The mice were swum twice daily for twenty minutes per swim. The swim-training was increased in 10-minute increments daily, until 90 minutes was reached, twice daily, by the end of the second week. Ninety-minute swims were maintained until the end of the study. Age- and weight-matched sedentary control mice were maintained and handled daily. Groups of control and swim-trained mice were killed after one, two, and four weeks of study. The heart weights and body weights of all mice were then determined. Two out of the 36 swim-trained mice were unable to complete the swim-training, but all other mice were conditioned successfully. At the conclusion of four weeks of exercise, the swim-trained mice developed a significant increase in HW:BW (16%) compared to that of the control mice ($p < 0.001$).

In McMullen's study (2003), groups of 14-16, 8-10-week-old male nontransgeneic (Ntg) or dominant negative PI3K (dnPI3K) transgenic FVB/N mice were swum in water tanks for four

weeks as described by Kaplan (1994), except that mice were trained seven days a week instead of five *(5)*. The HW:BW of Ntg mice subjected to swimming increased by ≈40%, whereas the HW:BW of dnPI3K transgenics increased by only 20% in response to swim training. McMullen found that this evidence supports the claim that PI3K(p110α) plays a more crucial role for the development of physiological than pathological cardiac hypertrophy.

In our first swim-training model, mice were swum in a tank of room-temperature water. Compared to Kaplan's study, which used a water temperature between 30-32°C, our swim tanks were too cold for the mice. Many mice began to die because of the cool water temperature so the experiment was terminated.

In our second swim-training model, nine female C57BL/6 mice were swum in 30-31°C tank, beginning with five minute swims. The same type of mice and the same water temperature from Kaplan's study were used in our experiment. However, we began with a shorter time interval for the swim-trained mice, increasing 10 minutes every other day, rather than increasing 10 minutes every day as was performed in Kaplan's study. We also had a much smaller 'n' in our study, with only nine swim-trained and seven control mice being used. Thirty-six swim-trained and 36 control mice were used for Kaplan's study. As the number of mice in the swim tank decreased, the mice seemed more likely to sink or float instead of swimming vigorously. Four swim-trained mice died possibly due to the rigorous swim-training or the lack of stimulation from the other mice. Because women have significantly greater amounts of total body fat than do men *(33)*, our assumption is that the female mice are able to float easier than the male mice due to their extra layer of fat. The fact that female mice were used rather than male could have been a factor in the lack of statistical significance for the difference in HW:BW seen in the control vs. swim-trained groups. All of the swim-trained mice had bloody lungs upon

dissection whereas none of the control mice showed this characteristic. The swim-trained mice may have had bloody lungs due to swallowing water as they swam each day or because of the increased time it took for the swim-trained mice to die by carbon dioxide gas.

In our third swim-training model, fifteen male C57BL/6 mice were swum according to swim-training model number two. Six sedentary mice were used. However, only six out of the 15 swim-trained mice completed the training. The veterinarian indicated that a respiratory infection could have caused several of these mice to die. We hoped that increasing the 'n' for the swim-trained mice would enable us to witness physiologic hypertrophy. A significant increase in HW:BW was observed for the swim-trained mice. However, when compared to Kaplan's study, which used 36 swim-trained mice, the number of swim-trained mice used to determine HW:BW in our experiment may not have been large enough to provide significant data for the phosphorylation states of the proteins under study. In the future, an even larger 'n' should be used to complete the swim-training. All of the swim-trained and control mice had bloody lungs upon dissection possibly due to the CO_2 used to kill them. Our speculation is that the males had to swim much harder to stay afloat compared to the effort exerted by the female mice in model number two because females tend to have a higher body fat percentage than do males *(33)*. The fact that male mice were used in this study could have been the key factor in the development of hypertrophy in the swim-trained group. The heart homogenates from the third swim-training model were used for protein analyses.

Akt and p70S6K were studied in this project because they are two of the downstream effectors in the PI3K pathway involved in hypertrophy of the heart. Akt is a kinase involved in cell survival and cell growth *(13)*. p70S6K regulates cell growth and plays a role in the pathway to determination of cell size *(16, 31)*. The development of hypertrophy in the swim-trained mice

was hypothesized to have arisen from the phosphorylation of Akt and/or p70S6K. However, there was no statistically significant difference between the phosphorylation states of Akt in the swim-trained vs. control mice. There also was no statistically significant difference in the phosphorylation states of p70S6K in the swim-trained vs. control mice. Akt and p70S6K were not phosphorylated to the degree expected possibly because PI3K is involved in so many other pathways. One of the catalytic subunits of PI3K, p110, has four isoforms (α, β, γ, δ). Perhaps there is another PI3K pathway involving a different isoform that caused the hypertrophy seen in the swim-trained mice.

An unanswered question in cardiac biology is whether different pathways are responsible for the development of physiologic and pathologic hypertrophy of the heart seen in humans *(5)*. Miyamoto found that the phosphorylation of p70S6K is regulated by an alternative pathway other than Akt in physiologic hypertrophy *(29)*. The results from Miyamoto's study indicate that pressure and volume overload-induced cardiac hypertrophy is associated with different patterns of activation of signal transduction pathways. In other words, two different types of stimuli will trigger a different type of pathway to become activated. One type of stimulus is high blood pressure, which can lead to heart disease. Another type of stimulus is vigorous cardiovascular training, which can lead to healthy enlargement of the heart. Therefore Akt and/or p70S6K may be phosphorylated in pathologic hypertrophy but not in physiologic hypertrophy.

Discrepancies. The results from our study indicate that Akt and p70S6K are not involved in the development of physiologic hypertrophy. In contrast, cardiac-specific overexpression of constitutively active Akt in mice led to hypertrophy of the heart in a different study, showing that Akt seems to be a key downstream effector of PI3K in the regulation of cardiac growth *(6)*. Also, a dominant negative p110αPI3K inhibits Akt activity and reduces heart size *(32)*.

In addition to these studies, McMullen found that phosphorylation of Akt and p70S6K were elevated at some points during the development of swimming induced-hypertrophy *(5)*. The western blot for McMullen's study shows much darker bands for the phosphorylated proteins in the swim-trained mice compared to the control mice. There was a significant difference in the phosphorylation state of Akt between the swim-trained and control mice after one week and three weeks of training. McMullen also saw a significant difference in the phosphorylation state of p70S6K between the swim-trained and control mice after one week and two weeks of training. It is interesting to note that there was not a significant difference in the phosphorylation states of Akt or p70S6K between the swim-trained and control mice after four weeks of study. In our experiment, the phosphorylation states of Akt and p70S6K were only determined after three-four weeks of training at 90 minutes, twice daily. Perhaps if we were able to separate the mice in our study into similar groups (one week, two weeks, three weeks, and four weeks of training), we may have seen a difference in the phosphorylation states of Akt and/or p70S6K throughout the duration of swim-training.

The data from Molkentin reveals yet another discrepancy *(34)*. Molkentin's study tested several signaling pathways in the hearts of patients with advanced heart failure, patients with compensated hypertrophy, and normal subjects. "Compensated hypertrophy" signifies that the patient had no history of heart failure, but upon echocardiograhy displayed marked cardiac hypertrophy, as evidenced by two-dimensional echo wall thickness. The signaling pathway involving Akt was one of the many studied in Molkentin's work. Activation of Akt has been associated with protection from apoptosis in heart cells *(35)*. Because progressive myocyte apoptosis may contribute to heart failure, Molkentin examined the phosphorylation status of Akt *(34)*. They observed a drastic increase in Akt phosphorylation in failing hearts compared with

control hearts as seen in the Western blots. The Akt phosphorylation status was insignificant in both hypertrophied hearts and control hearts. Their data indicate that the PI3K/Akt signaling pathway is significantly activated in failing hearts, but not activated in hypertrophied hearts. Molkentin's report contains the first data that associates Akt activation with heart failure.

Because Molkentin witnessed a striking difference in the activity of signaling pathways between patients with compensated hypertrophy and those with heart failure, it is very possible that there are distinct signaling pathways involved in volume overload-induced hypertrophy (healthy growth) and pressure overload-induced hypertrophy (unhealthy growth). If there is a difference in the signaling pathways between physiologic and pathologic hypertrophy, our data may be stating the truth – that there is no Akt activation in physiologic hypertrophy. As Molkentin observed in humans, our mice may have displayed Akt activation had we tested mice with heart failure, rather than enlarged healthy hearts.

Lastly, one of the main discrepancies in our study was the fact that the 'n' became very small throughout the swim-training. A larger population is needed in order to differentiate the pathways involved in physiologic and pathologic hypertrophy of the heart. Perhaps if we had a larger 'n', we may have seen a significant difference in the phosphorylation states of Akt and/or p70S6K. But, it is also possible that there truly is no difference in the phosphorlyation states of Akt and p70S6K seen in physiologic hypertrophy. In our study, these proteins appeared to have no significant impact on physiologic hypertrophy. However, Akt and/or p70S6K may be involved in pathologic hypertrophy if, indeed, there are different pathways involved for physiologic and pathologic hypertrophy of the heart.

Conclusions. Most female mice did not develop hypertrophy to the degree that males did. The third swim-training model that used male C57BL/6 mice was successful in inducing cardiac

hypertrophy. Physiologic hypertrophy is not associated with the phosphorylation of either Akt or p70S6K.

Future Directions. Understanding the regulation of protein pathways is essential for the progression of research on the number one killer in the United States, heart disease. Even though physiologic cardiac hypertrophy was successfully induced in mice, protein analyses did not support the hypothesis that the phosporylation of Akt and p70S6K are involved in this process. There have been some discrepancies within the research on PI3K and its downstream effectors. Therefore, more research is needed to determine the protein pathways involved in pathologic hypertrophy and physiologic hypertrophy. More research also is needed to determine the signaling pathways involved in the progression from pathologic hypertrophy to a failing heart.

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